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Identification, Cloning, and Sequencing of *piv*, a New Gene Involved in Inverting the Pilin Genes of *Moraxella lacunata*

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Moraxella lacunata **is a bacterium that is a causative agent of human conjunctivitis and keratitis. We have previously cloned the Q and I pilin (formerly called β and α pilin) genes of *Moraxella bovis* and determined that an inversion of 2 kilobases (kb) of DNA determines which pilin gene is expressed. Using an *M. bovis* pilin gene as a hybridization probe to screen a λ ZAP library of *M. lacunata* DNA, we have isolated a clone that not only contains the entire type 4 pilin gene inversion region of *M. lacunata* but inverts the 2-kb region on a plasmid subclone (pMxL1) in *Escherichia coli*. Deletion derivatives of pMxL1 yielded some plasmids that still had the entire inversion region but were phase locked into one or the other of the two potential orientations. Similarly, insertions of a 2-kb streptomycin-resistant element (Ω) within some regions outside of the inversion also resulted in phase-locked plasmids. These deletions and insertions thus localize a probable invertase necessary for the inversion event. The region was sequenced, and an open reading frame with over 98% DNA sequence homology to an open reading frame that we previously found in *M. bovis* and called ORF2 appeared to be a strong candidate for the invertase. This conclusion was confirmed when a plasmid containing the *M. bovis* ORF2 supplied, in *trans*, the inversion function missing from one of the *M. lacunata* phase-locked inversion mutants. We have named these putative invertase genes piv_{ml} (pilin inversion of *M. lacunata*) and piv_{mb} (pilin inversion of *M. bovis*). Despite previously noted sequence similarities between the *M. bovis* sites of inversion and those of the Hin family of invertible segments and a 60-base-pair region within the inversion with 50% sequence similarity to the *cin* recombinational enhancer, there is no significant sequence similarity of the Piv invertases to the Hin family of invertases.**

Members of the type 4 (MePhe) class of bacterial pili are found on a wide variety of gram-negative bacteria (9), including *Moraxella bovis* (25), *Moraxella nonliquefaciens* (12), *Neisseria gonorrhoeae* (15, 36), *Neisseria meningitidis* (15), *Bacteroides nodosus* (28), *Pseudomonas aeruginosa* (35), and *Vibrio cholerae* (43). The main structural subunits of these pili (pilins) share extensive amino-terminal amino acid sequence homology and all except *V. cholerae* contain the modified amino acid *N*-methylphenylalanine as the first residue of the mature protein (15, 28). The functional significance of this conservation has been demonstrated by the ability of *P. aeruginosa* bacteria containing plasmids expressing the *B. nodosus* pilin gene to process and assemble the *B. nodosus* pilins into pili on the *P. aeruginosa* cell surface which are structurally and immunologically indistinguishable from authentic *B. nodosus* pili (10, 27). Recently, similar experiments in *P. aeruginosa* that contained an *M. bovis* pilin gene produced bacteria that expressed chimeric pili containing both *M. bovis* pilin and *P. aeruginosa* pilin (3).

Both similarities and differences in the genetic organization of type 4 pilin genes occur in different bacterial species. One similarity is that *P. aeruginosa*, *N. gonorrhoeae*, and *M. bovis* pilin genes all appear to use *rpoN* (*glnF*, *ntrA*)-dependent promoters (18, 19). RpoN has been shown to be the alternative sigma factor, σ^{54}, required for transcriptional activation of some genes (reviewed in reference 22). Differences exist in the copy numbers of pilin genes among type 4 pili. *P. aeruginosa* strains only have a single copy of the pilin gene in each genome (29, 35). Most serotypes of *B. nodosus* only have a single gene, but some are organized with the genes *fimA*, *fimC*, *fimZ* in order and transcribed in the same direction, where *fimA* and *fimZ* are both pilin genes (26). *N. gonorrhoeae*, by contrast, contains multiple pilin gene loci in every strain, and transitions from P^+ to P^- and between different P^+ pilin types are often accompanied by chromosomal DNA rearrangements (reviewed in reference 38). Most strains each have a single pilin expression locus (42) and multiple silent (incomplete) variant pilin sequences lacking the common N-terminal coding sequence of pilin (38). Antigenic variation from one P^+ type to another is a result of transformation of chromosomal DNA from other gonococci that have autolyzed, followed by a $recA^+$-dependent recombination event between silent-copy donor DNA and the recipient expression locus (38).

We are studying the pilin gene regions of *M. bovis* and *M. lacunata*. *M. bovis* and *M. lacunata* are very closely related, as determined by both DNA-DNA hybridization analysis (44) and transformation studies (4, 5). *M. bovis* is an ocular pathogen of cattle, and *M. lacunata* is a pathogen that occasionally causes conjunctivitis in humans (1, 2, 8, 31). A single strain of *M. bovis* is capable of producing one or the other of two pilin types, which are now called Q and I (formerly β and α) for strain Epp63 (25, 32). The switch in expression between Q and I pilin is due to an inversion of a 2.1-kilobase (kb) region of DNA (13, 24).

Among the best-characterized families of bacterial DNA inversion systems are the Hin system of *Salmonella typhimurium*, Gin and Cin of bacteriophages Mu and P1, and Pin of *Escherichia coli* (reviewed in reference 14); another is the newly described Min of plasmid p15B (33). We have previously shown that the *M. bovis* pilin gene inversion recombi-

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national sites show sequence similarity to those of the Hin family of invertible segments (13, 24) and that a region within the inversion has sequence similarity to the recombinational enhancer sequences found in the Hin family (13).

In this report, we describe the cloning of an *M. lacunata* pilin gene region which contains a 2-kb segment that inverts on a plasmid subclone in *E. coli*. We identify and present the sequence of a gene, *piv*, which is the probable invertase, and show that unlike the other elements of the system, which have sequence similarity to the Hin family, *piv* has no sequence similarity to the *hin*, *cin*, *gin*, or *pin* recombinase genes.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. *M. bovis* Epp63 and the recombinant plasmids pMxB12 and pMxB19 were described previously (13, 25). *M. bovis* was grown on GC agar base (Difco Laboratories, Detroit, Mich.) with 1% IsoVitaleX (BBL Microbiology Systems, Cockeysville, Md.). *E. coli* strains containing drug-resistant plasmids were grown on L agar containing the following concentrations (in micrograms per milliliter) of the appropriate antibiotic: carbenicillin, 100 (LBcb); streptomycin, 100 (LBsm), spectinomycin, 25 (LBsp); or tetracycline, 15 (LBtet) (Sigma Chemical Co., St. Louis, Mo.). *E. coli* BB4 [*supF58 supE44 hsdR514 galK2 galT22 trpR55 metB1 tonA* Δ(*argF-lacZYA*)*U169* (F′ *proAB lacI*^{q}ZΔM15 Tn*10*)] (6) was used for growing and screening the λ ZAP library on NZCM agar plates (37). *E. coli* DH5α [F$^-$ *endA1 hsdR17 supE44 thi-1 recA1 gyrA96 relA1* Δ(*arg*F-*lacZYA*)U169 (φ80d *lacZ*ΔM15)] (Bethesda Research Laboratories, Inc., Gaithersburg, Md.) was used in transformation of the deletion derivatives of pMxL1 and the Ω insertion derivatives of dR13. *E. coli* MC1061 [*hsdR araD139* Δ*araABC* Δ(*olc-leu-7679*) *galU galK* Δ*lacX74 aspL thi*] (7) was used in the in vivo Hin complementation experiments. *E. coli* EB1928 [*endA thi-1 hsdR17 supE44* Δ*lacU169*, Δ(*glnF*::Tn*10*)] was kindly provided by Robert Bender, University of Michigan, Ann Arbor. *M. lacunata* ATCC 17956 was kindly provided by Elliot Juni, University of Michigan, Ann Arbor, and was grown on heart infusion agar at 35°C. Plasmid pHP45 Ω was generously provided by Henry M. Krisch, Université de Genève, Geneva, Switzerland.

DNA isolation, manipulation, and sequencing. Total DNA was prepared by the method of Hull et al. (17). Phage and plasmid DNAs were isolated as described previously (25). Restriction endonucleases were purchased from New England BioLabs, Inc., Beverly, Mass.; Bethesda Research Laboratories, Inc.; Boehringer Mannheim Biochemicals, Indianapolis, Ind.; or Promega Biotec, Madison, Wis. Restriction endonuclease digestions and agarose gel electrophoresis were carried out as previously described (25). Isolation of restriction enzyme-generated DNA fragments from ethidium bromide-stained gels was carried out by using long-wave UV light to localize the fragments; then the bands containing the fragments were excised from the gel, and the agarose was removed by using GeneClean (Bio 101, Inc., La Jolla, Calif.). DNA transfer from agarose gels to nitrocellulose paper was done by the method of Southern (41), and hybridizations were done as previously described (25). DNA fragment probes were labeled with [α-^{32}P]dCTP (Dupont, Research Products, Boston, Mass.) by use of nick translation kit (Bethesda Research Laboratories, Inc.).

A λ ZAP library was constructed for us (Stratagene, San Diego, Calif.) by using genomic DNA derived from piliated *M. lacunata* ATCC 17956. The library was screened by using a nick-translated *Eco*RI-*Hin*dIII fragment of pMxB12 as a probe according to previously described procedures (25). Positive plaques were purified, and then in vivo subcloning was carried out by superinfection with helper phage R408 as described by Short et al. (40). One resulting subclone containing the *M. lacunata* pilin gene inversion region was named pMxL1. Deletion derivatives of pMxL1 were made by making a single cut at the *Cla*I and *Apa*I sites in the pBluescript vector. Exonuclease III digestion of the double-cut DNA from the 5′ overhang was carried out by using the Erase-a-Base system (Promega). The deleted DNAs were ligated and transformed into *E. coli* DH5α, and transformants were selected on LBcb plates. Mini-plasmid preps (23) were performed on drug-resistant transformants, and the size of each deletion was determined by cutting the DNA with a uniquely cutting restriction enzyme and running the samples on 1% agarose gels.

Ω interposon variants of pMxL1dR13 were constructed as follows. pMxL4 was created by ligating pMxL1dR13 cleaved with *Hin*dIII to the Ω fragment isolated from pHP45 Ω by cleavage with *Hin*dIII. pMxL5 and pMxL6 were made by ligating pMxL1dR13 cleaved with *Hin*cII to the Ω fragment isolated from pHP45 Ω by blunt-end cleavage with *Sma*I. pMxL7 and pMxL10 were created by cleaving pMxL1dR13 with *Acc*I, filling in the ends with the Klenow fragment by the method of Maniatis et al. (23), and then ligating the ends to the Ω fragment with *Sma*I blunt ends. To construct pMxL8, pMxL1dR13 was first cleaved with *Sma*I and *Sph*I; the ends were filled in as described above and ligated, resulting in molecules deleted for a small amount of the left end of the insert as well as for the left *Eco*RI site and the *Pst*I site. These molecules were then redigested with both *Eco*RI and *Pst*I (to eliminate molecules that had not been deleted properly), treated with bacterial alkaline phosphatase, and ligated to the Ω fragment with *Eco*RI ends.

Deletion derivatives of pMxL1 were sequenced by the dideoxy-chain termination method of Sanger et al. (34) as previously described (13) and also by using the synthetic primers L1 (5′-CGCCCACTACACGAGTAG-3′) and L2 (5′-GCGGGATATCGACCAAAGC-3′), whose positions are shown in Fig. 3. Sequence analysis was performed by using computer software from DNASTAR, Inc., Madison, Wis.

In vitro Hin inversion assay. In vitro inversion assays were performed essentially as described by Johnson et al. (20). A 0.5-μg sample of phase-locked DNA of plasmid pMxL1dL19 or pMxL1dL24, which are phase locked in different orientations, was incubated with 100 or 200 ng of Hin, 30 or 60 ng of Fis, and 100 or 200 ng of HU in 0.1 M NaCl–1 mM dithiothreitol–10 mM $MgCl_2$, 20 mM Tris hydrochloride (pH 7.5)–100 μg of polycytidylic acid per ml. Plasmid pMS504 was used as a positive control (20, 21).

In vivo Hin inversion assays. *E. coli* MC1061 was transformed separately with pMxL1dL19, pMxL1dL24, and pMS504, and each of these plasmids was cotransformed into MC1061 with pKH66, which carries the *hin* gene under control of the *tac* promoter and *lacI*^{q} (16). Transformants of MC1061 with pMxL1dL19 and pMxL1d24 were plated on LBcb, transformants of pMS504 were plated on LBtet, and cotransformants with pKH66 were plated on LBcb-sp or LBtet-sp. After the transformants were restreaked on the appropriate LB antibiotic plates, liquid cultures were grown from single colonies in L broth containing the appropriate antibiotics at 37°C to stationary phase and then diluted 100-fold in fresh medium. These cultures were grown to an optical density at 600 nm of 0.5, 3 ml of each culture was

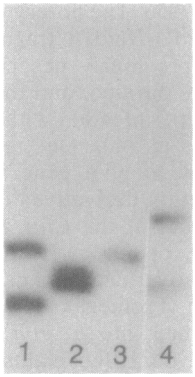

FIG. 1. Genomic Southern hybridizations using an *M. bovis* Q pilin gene probe and *Hin*fI-digested genomic DNA. Lanes: 1, piliated *M. lacunata* ATCC 17956; 2, nonpiliated *M. lacunata* ATCC 17956; 3, Q piliated *M. bovis* Epp63; 4, I piliated *M. bovis* Epp63.

removed for plasmid isolation, and the cultures were then diluted fivefold in fresh medium containing 4×10^{-5} or 4×10^{-4} M isopropyl-β-D-thiogalactopyranoside (IPTG). After 90 min, 3 ml of each of these induced cultures was used for plasmid isolation. The cultures were diluted threefold in fresh IPTG-containing medium and grown overnight. Plasmid DNA was then isolated from the overnight cultures.

Plasmid DNA isolated from uninduced, 90-min induction, and overnight induction cultures were digested with *Kpn*I for pMxL1dL19 and pMxL1dL24 and with *Cla*I for pMS504 before agarose gel electrophoresis.

Complementation of pMxL5 by *M. bovis* ORF2. *E. coli* DH5α cells containing pMxL5 were transformed with pMxB20, an open reading frame 2 (ORF2)-containing plasmid with ampicillin and tetracycline resistance markers (25), plated on LBtet, and grown overnight at 37°C. As a control to the experiments described in Results, 200 colonies of pMxL5 were screened for their ability to invert in the absence of a complementing plasmid, and none did.

RESULTS

Cloning of the *M. lacunata* pilin gene region. As has been previously described (24), *M. bovis* DNAs isolated from Q or I pilin-producing bacteria and cleaved with *Hin*fI give different genomic Southern hybridization patterns when probed with a region of pilin gene DNA (Fig. 1). These differences are due to an inversion event that regulates pilin expression. While studying the patterns of hybridization of the *M. bovis* Q pilin gene to *M. lacunata* genomic DNAs, we observed that one of the nonpiliated variants of strain ATCC 17956 had a hybridization pattern different from that of its piliated parent (Fig. 1). Because these different patterns looked very similar to those seen between Q and I DNAs of *M. bovis*, we decided to isolate and characterize the *M. lacunata* pilin gene region.

After obtaining a λ ZAP library of piliated ATCC 17956 genomic DNA, we used our cloned *M. bovis* Q pilin gene (25) as a hybridization probe and screened approximately 34,000 plaques, picking 13 potential positives. After rescreening and plaque purification, one of the positives was subcloned by an in vivo method, using an M13 helper phage (40). One of the resulting plasmid clones (pMxL1) was then isolated for further characterization.

Characterization of pMxL1 and construction of deletion and insertion derivatives. Restriction analysis of pMxL1 with a variety of enzymes gave a size estimate of 8.9 kb (Fig. 2A), with the pBluescript vector portion representing 2.95 kb of the total. A different result occurred when pMxL1 was cleaved with *Kpn*I, which resulted in the production of four fragments with sizes of 7.5, 5.8, 3.2, and 1.4 kb (Fig. 2B). The sum of the sizes of these four fragments is 17.9 kb twice that obtained by the other enzymes tested. Furthermore, the sum of the sizes of the largest and smallest *Kpn*I fragments is 8.9 kb, whereas the sum of the sizes of the internal fragments is 9.0 kb. This result suggested that an inversion event was occurring in plasmid pMxL1 in *E. coli*, with a *Kpn*I site present asymmetrically within the invertible region. The plasmid appears to be twice as big in a *Kpn*I digest because the plasmid DNA being cleaved is actually a mixture of two plasmid DNA types, each having the invertible segment in a different orientation. The resulting restriction map of pMxL1 is shown in Fig. 3A (orientation 1) and Fig. 3B (orientation 2).

We next constructed deletion derivatives of pMxL1 as described in Materials and Methods. Linearization of each deletion derivative by *Xho*I cleavage is shown in Fig. 2A, and the region deleted in each is graphically illustrated in Fig. 3. *Kpn*I cleavage of the left-end deletion derivatives revealed that two of them (pMxL1dL17 and pMxL1dL10) still produced four bands, implying that like pMxL1, they were still capable of inversion of the 2-kb invertible segment (Fig. 2B). However, starting with pMxL1dL19 (whose deletion endpoint lies between the internal *Eco*RI site and the internal *Hin*cII site), the smaller left-end deletion plasmids only had two bands (Fig. 2B). Several of the left-end deletion derivatives were phase locked despite containing the entire inversion region plus at least 1 kb of DNA to the left of the inversion (Fig. 3). This finding implies that some function necessary for the inverting of the inversion region on plasmids in *E. coli* has been lost or made inoperable in these deletion derivatives. To investigate this question further, we constructed insertion mutations by using the Ω interposon, an element which contains a streptomycin-spectinomycin resistance gene as well as transcriptional and translational stop signals and is flanked by linkers with restriction sites to facilitate cloning (11, 30). We placed the Ω fragment at the internal *Hin*dIII, *Eco*RI, *Hin*cII, and *Acc*I sites of pMxL1dR13 (for details, see Materials and Methods; positions of the insertions are shown on Fig. 3). Constructs with the Ω fragment inserted at either the *Hin*dIII (pMxL4) or *Eco*RI (pMxL8) site resulted in plasmids still capable of inverting the inversion region, but insertions at either the *Hin*cII (pMxL5 and pMxL6) or *Acc*I (pMxL7 and pMxL10) site produced noninvertible phase-locked plasmids.

In vitro and in vivo Hin complementation assays. We have discovered sequence similarities between the *M. bovis* pilin gene inversion region and the Hin family of invertible segments both at the actual sites of inversion (24) and at a sequence called the recombinational enhancer (13). Therefore, we wanted to determine whether the *Moraxella* pilin gene inversion system and the Hin inversion system were related enough functionally to complement each other. The in vitro Hin inversion system was tested for its ability to invert the phase-locked plasmids pMxL1dL19 and pMxL1dL24 (one locked in each orientation). However, even when up to a 10-fold excess of Hin was used compared with that needed to invert the Hin region plasmid pMS504 (used as a positive control), no evidence of any inversion of plasmid pMxL1dL19 or pMxL1dL24 was observed (data not shown). Similarly, in vivo complementation of pMxL1dL19 and pMxL1dL24 with Hin provided in *trans* from pKH66,

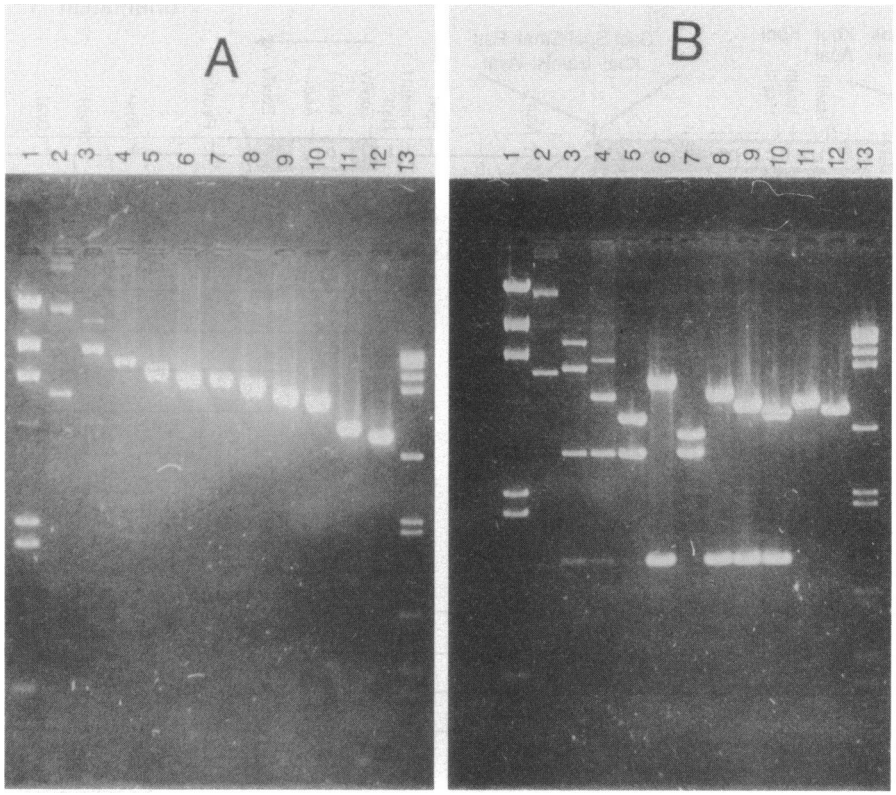

FIG. 2. (A) *Xho*I-digested plasmid DNA electrophoresed in 1% agarose gels. Lanes: 1, λ cut with *Hin*dIII; 2, pMxL1 uncut; 3, pMxL1; 4, pMxL1dL10; 5, pMxL1dL19; 6, pMxL1dL24; 7, pMxL1dL25; 8, pMxL1dL34; 9, pMxL1dL38; 10, pMxL1dL43; 11, pMxL1dL12; 12, pMxL1dL44; 13, λ cut with *Dra*I. (B) Lanes: 1, 2, and 13, same as in panel A; 3 to 12, same plasmid DNAs as in panel A but cleaved with *Kpn*I instead of *Xho*I.

which has the *hin* gene under control of the *tac* promoter (16), resulted in no inversion even at induced Hin levels that were about 10-fold higher than the induced levels which gave inversion of the control phase-locked plasmid pMS504 (data not shown).

Sequence analysis of the gene on pMxL1 essential for inversion. Having localized a region essential for the invertibility of the 2-kb pilin gene region of pMxL1, we sequenced the region (Fig. 4) by dideoxy sequencing of various pMxL1 deletion derivatives (see Materials and Methods for details). The DNA sequence of this region of *M. lacunata* DNA is very similar to the region of *M. bovis* DNA that encodes the previously described *M. bovis* pilin gene region ORF2 (13). Figure 4 also shows the predicted amino acid sequence of the *M. bovis* ORF2 below that of the *M. lacunata* ORF. The predicted amino acid sequences are highly conserved, with only 5 differences out of 322 amino acids. At the DNA sequence level there are 12 of 969 base pair differences within the ORFs, and all 113 base pairs immediately 5′ of the ORFs are identical.

Complementation of pMxL1 inversion by *M. bovis* ORF2. Data from both deletion derivatives and insertion mutants of pMxL1 are consistent with the hypothesis that the *M. lacunata* ORF described above is the function missing in those pMxL1 derivatives which are phase locked. Figure 3 lists the insertions and deletions still capable of inversion and those that are phase locked. Insertions of Ω into either the *Hin*dIII site or the *Eco*RI site, both of which are just 5′ of the ORF, resulted in plasmids that still inverted, whereas insertions into sites within the ORF (*Hin*cII and *Acc*I) resulted in phase-locked plasmids. Similarly, left-end deletion derivatives were still capable of inversion until they entered the ORF. One hypothesis for this result is that the *M. bovis* ORF2 and the *M. lacunata* ORF encode the site-specific invertases required for pilin gene inversion.

To test this hypothesis, pMxB20, which is a tetracycline-ampicillin-resistant pBR322 derivative containing the *M. bovis* ORF2, was transformed into competent *E. coli* cells containing pMxL5, a phase-locked Ω insertion mutant of pMxL1 which is incompatible with pMxB20. After selection for entry of pMxB20 by plating cells on LBtet, colonies were restreaked on LBsm plates to select for the presence of pMxL5. We picked 32 colonies from the LBsm plates and patched them onto both an LBsm and an LBtet plate. All grew on both plates. We restreaked colonies from the LBsm patches onto LBsm plates two more times and then again patched all 32 onto LBtet and LBsm. After these additional passages, 12 of 32 grew only on LBsm, not on LBtet. These 12 were grown, and plasmid DNA was isolated from them. The *Kpn*I digestion patterns of these 12 revealed that 9 had the original orientation 1 pattern of pMxL5 and that the other 3 were a mixture of orientations 1 and 2. One of these three mixed strains was again restreaked on LBsm; 10 colonies were picked and grown, and plasmid DNA was isolated. Of these 10, 4 contained plasmids only in orientation 1, 4 were still mixed, and 2 had resolved to only orientation 2. Once a strain was only expressing orientation 1 or orientation 2 it was stable, and further growth did not result in any plasmids in the alternative orientation in the same cell. Given this evidence that *M. bovis* ORF2 had acted as a *trans*-acting invertase to invert the phase-locked pMxL5 into the orien-

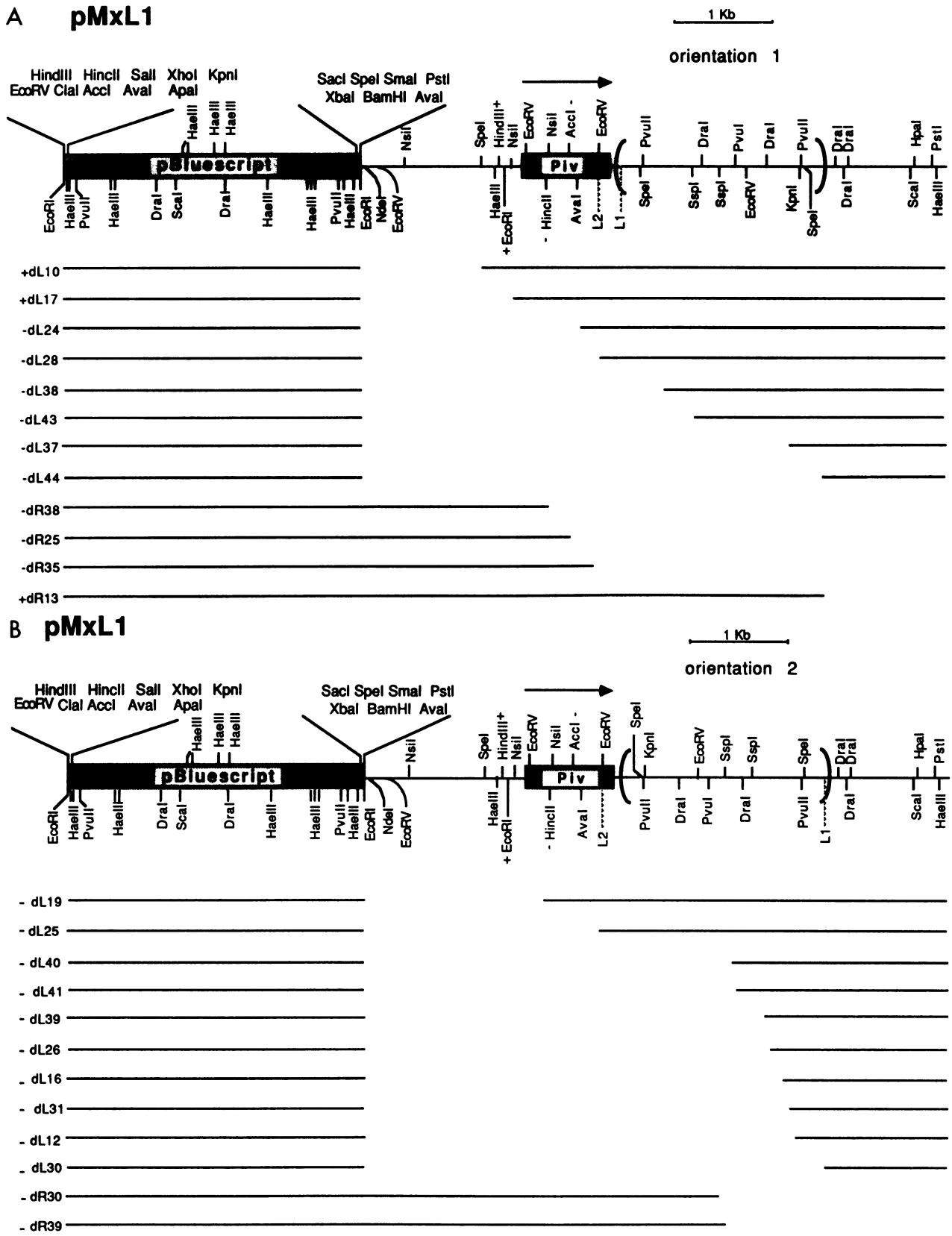

FIG. 3. (A) Restriction map of plasmid pMxL1 and deletion constructs in orientation 1 as determined by *Kpn*I restriction digestion profiles or by DNA sequence analysis. L1 and L2 show sites of oligonucleotide primers used in DNA sequence analysis. The + signs at the internal *Eco*RI and *Hin*dIII sites indicate where insertion of the 2-kb Ω interposon results in plasmids still capable of inverting; the − signs at the internal *Hin*cII and *Acc*I sites indicate where Ω insertions result in phase-locked plasmids. Extent of DNA remaining in the deletion derivatives is shown by solid lines below the restriction map. A + sign in front of the deletion derivative means that it is still capable of inversion; a − sign in front means that it is phase locked in orientation 1. (B) Restriction map of plasmid pMxL1 in orientation 2. The deletion derivatives represented below the map are all phase locked in orientation 2.

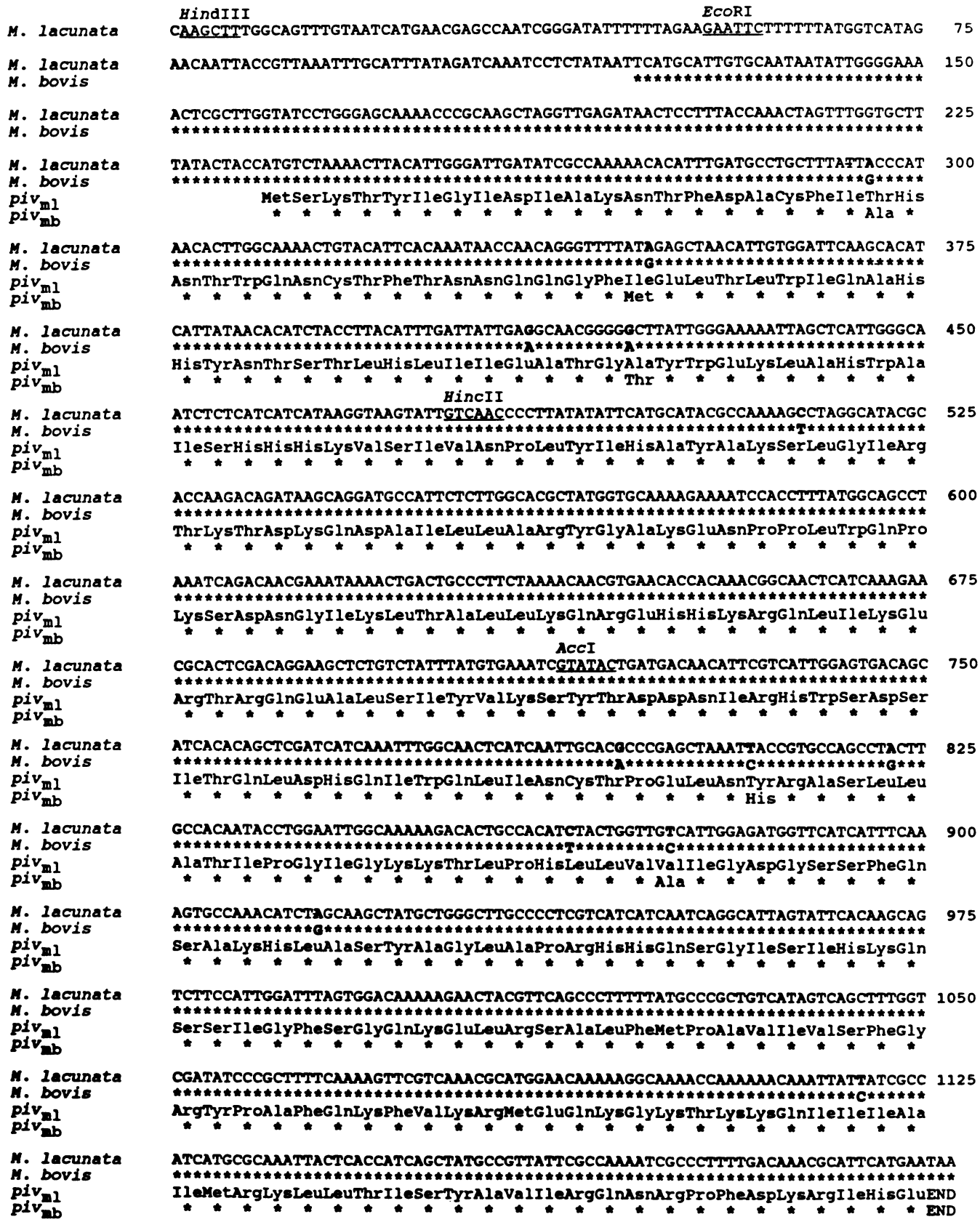

FIG. 4. Comparison of piv_{ml} and piv_{mb} DNAs and amino acid sequences. *, Identity to either the above-listed nucleotide or amino acid. The four restriction sites at which the Ω interposon was inserted are underlined, with the enzyme name above. These nucleotide sequence data have been submitted to GenBank, the *M. lacunata* sequence assigned accession number M34367 and the *M. bovis* sequence from Fulks et al. (13) assigned accession number M32345.

tation seen in pMxL6, we named it piv_{mb}, and the *M. lacunata* gene was named piv_{ml}.

Invertibility of pMxL1 in a σ^{54} mutant *E. coli*. We tested whether piv_{ml} uses a σ^{54}-dependent promoter by transforming pMxL1 into the *E. coli glnF* deletion strain EB1928. *Kpn*I digestion and agarose gel analysis of pMxL1 plasmid DNA isolated from this strain showed that the 2-kb region still inverted (data not shown).

DISCUSSION

We have cloned the pilin gene inversion region into *E. coli* from piliated *M. lacunata* ATCC 17956 from a λ ZAP genomic library, using an *M. bovis* pilin gene as a probe. One resulting plasmid subclone (pMxL1) was found to invert a 2-kb region of DNA. Construction of deletion and insertion derivatives of pMxL1 revealed that a function situated immediately adjacent to the invertible region was essential for the inversion event. Sequence analysis of this adjacent region revealed an ORF that differs from the previously described ORF2 of the *M. bovis* pilin gene region in only 5 of 322 amino acids. These genes seemed likely candidates for the site-specific invertases; to test this possibility, we used a plasmid containing the *M. bovis* ORF2 region to transcomplement pMxL5, a phase-locked Ω insertion mutant of pMxL1. Since this attempt was successful, we named ORF2 of *M. bovis* piv_{mb} and the corresponding gene in *M. lacunata* piv_{ml}.

We do not know the position of the transcriptional start site of either *piv* gene, nor for certain the translational start sites. When we first reported the sequence of *M. bovis* ORF2 (piv_{mb}), we noted potential σ^{54} binding sites at the start of the gene (13). The region containing these potential σ^{54} promoter sites of piv_{mb} is identical in sequence in *M. lacunata*. We tested the ability of pMxL1 to invert in an *E. coli* deletion mutant lacking σ^{54}. Since the 2-kb region of pMxL1 still inverts in this σ^{54} mutant strain at a rate indistinguishable from that seen in a σ^{54} wild-type strain, σ^{54} is not required in order to obtain sufficient expression of *piv* to carry out the inversion event on pMxL1. Therefore, piv_{ml} does not appear to use a σ^{54}-dependent promoter. We plan to isolate *piv* mRNA and to map the actual transcriptional start sites of the *piv* genes by S1 mapping or by reverse transcriptase mapping in the near future.

Some of the interesting features of the *M. bovis* pilin gene inversion system that we noted previously are sequence similarities to the Hin family of invertible elements both at the recombinational inversion sites (13, 24) and at a potential recombinational enhancer sequence (13). As soon as we obtained phase-locked deletion derivatives of pMxL1 that still contained the entire 2-kb invertible element, we attempted to invert them by using the Hin in vitro inversion system. These attempts failed, as did the in vivo complementation assays with Hin provided in *trans* from a plasmid containing the *hin* gene under control of p_{tac} and $lacI^q$. Once we obtained the sequence of Piv_{ml}, we were able to compare the sequences of Piv_{ml} and Piv_{mb} with the sequences of Hin, Gin, Pin, and Cin (39). To our surprise, there is no apparent amino acid sequence similarity between either Piv and any of the Hin family of invertases. Similarly, we checked all other possible ORFs within the *piv* gene region (the largest of which was 63 amino acids), and none of them showed any amino acid sequence similarity to Hin-like invertases. Investigators have noted regions of amino acid sequence conservation between the Hin family of invertases and resolvases found in the Tn*3* family of transposable elements (39). When we use only short stretches of amino acid sequence that have been shown to be most highly conserved between both the Hin family of invertases and the Tn*3* family of resolvases, we still cannot find any regions of Piv similar to these sequences. Therefore, despite the previously noted sequence similarities between the Hin family and the *M. bovis* pilin gene inversion region, the invertases appear totally unrelated. In addition, there are no amino acid sequence similarities between Piv and the Int family of bacteriophage site-specific recombinases.

It should be made clear that although Piv is most likely directly involved as the invertase, further biochemical studies showing that the Piv protein actually binds to the inversion site sequences need to be carried out, since it could be that Piv is a protein which helps some indigenous *E. coli* invertase carry out the inversion process. It remains to be determined whether the same host elements used in Hin inversion systems (Fis and HU proteins) are needed for Piv-dependent inversion or whether other host proteins might be essential.

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